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CIGARETTE SMOKE DECREASES THE RATE CONSTANT FOR THE ASSOCIATION OF ELASTASE WITH α_1-PROTEINASE INHIBITOR BY A NON-OXIDATIVE MECHANISM

PHILIPPE LAURENT AND JOSEPH G. BIETH

INSERM UNITÉ 237, FACULTÉ DE PHARMACIE,
UNIVERSITÉ LOUIS PASTEUR,
B.P. 10
67048 STRASBOURG, FRANCE

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This paper describes a non-oxidative impairment of the biological function of α_1-proteinase inhibitor by cigarette smoke. Aqueous solutions of cigarette smoke are able to decrease the rate constant k_{ass} for the inhibition of porcine pancreatic elastase by human plasma α_1-proteinase inhibitor. The value of k_{ass} decreases linearly with the concentration of smoke (from 2.2×10^5 M^{-1} s^{-1} to 0.6×10^5 M^{-1} s^{-1}). This effect is not due to an oxidation of the inhibitor. When pancreatic elastase is reacted with elastin in the presence of α_1-proteinase inhibitor and cigarette smoke solution, elastolysis occurs at a rate nearly identical to that observed in the absence of inhibitor. This effect is due to a smoke-induced decrease in k_{ass}. These observations may serve as a model of biological regulation of proteolysis via a change in the rate constant for a proteinase-proteinase inhibitor association. The influence of cigarette smoke on the inhibition of human neutrophil elastase by α_1-proteinase inhibitor could not be studied in detail because the enzyme precipitates in the presence of concentrated smoke solution.

Pulmonary emphysema is thought to result from the action of elastases on lung elastin. Elastolysis is normally prevented by lung proteinase inhibitors such as α_1-proteinase inhibitor (α_1PI[1])(1). On the other hand, smoking is an important risk factor of emphysema. Cigarette smoke attracts neutrophils in the lung and releases neutrophil elastase locally (2). Oxidation of α_1PI is another biochemical link between smoking and emphysema (3,4). Oxidized α_1PI reacts 3000 times slower with neutrophil elastase than the native inhibitor (5). As a consequence, oxidized α_1PI is unable to prevent elastolysis, at least in an *in vitro* system (6). Inactive (oxidized) α_1PI was detected in bronchoalveolar lavage fluids from smokers (7,8). These findings were, however, contradicted (9,10) and there is no firm proof, as

[1] Abbreviation : α_1PI, α_1-proteinase inhibitor (α_1-antitrypsin)

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yet, that biological oxidation of α_1PI plays a significant role in the pathogenesis of smokers'emphysema.

This raises the question of whether cigarette smoke could not impair the elastase-α_1PI interaction by a non-oxidative mechanism e.g. by lowering the rate of elastase inhibition. Elastase (E) reacts with α_1PI according to the following scheme (5): $E + \alpha_1PI \xrightarrow{k_{ass}} E\text{-}\alpha_1PI$. In the absence of a competing substrate (elastin) the inhibition process will go to completion even if a component of cigarette smoke decreases k_{ass}. By contrast, when elastin is present, a moderate decrease in k_{ass} may significantly favor the binding of elastase to elastin i.e. there will be elastolysis despite the presence of α_1PI. We have therefore investigated the effect of cigarette smoke on the rate constants for the inhibition of pancreatic and neutrophil elastase by α_1PI and on the rate of elastolysis of elastin in the presence of α_1PI.

MATERIALS AND METHODS

Materials :

The elastase substrates succinyl-$(Ala)_3$-p-nitroanilide (11) Succinylmethylester-$(Ala)_2$-Pro-Val-p-nitroanilide (12) and remazol-brilliant-blue elastin (13) came from Choay (Paris), Bachem (Bubendorf, Suisse) and Elastin Product Co (St Louis, MO, USA), respectively. Stock solutions of the two synthetic substrates were prepared in N-methylpyrrolidone. Porcine pancreatic and human neutrophil elastases were isolated and active-site titrated by published procedures (14-16) α_1PI was a gift from Dr. James Travis (University of Georgia, Athens G.A., USA).

Preparation of aqueous cigarette smoke solutions

"Cigarette smoke solutions" were prepared from commercial, unfiltered cigarettes (trade name : Gitanes) using a device described by Carp and Janoff (3). Consecutive 20 ml puffs were slowly drawn from 2 cigarettes (15 seconds per puff) and bubbled immediatly into 3 ml of 0.02 M or 0.2 M Tris-base. The "smoke solutions" were then adjusted to pH 8.0 using 1 N NaOH and used immediately.

Measurement of the rate constant for the association of pancreatic elastase with α_1PI

Equimolar quantities of elastase and α_1PI (0.9×10^{-7}M) were added simultaneously to 0.2 M Tris-HCl pH 8.0 25°C containing variable proportions of "smoke solution". The total volume of the reaction medium was 300 µl. After selected time intervals, the association was stopped by the sequential addition of 5 µl of 100 mM succinyl-$(Ala)_3$-p-nitroanilide and 700 µl of 0.2 M Tris-HCl pH 8.0. The rate of substrate hydrolysis was measured kinetically at 410 nm and 25°C in a double-beam spectrophotometer. The reference cuvette contained the same reagents as the test cuvette except the enzyme.

Kinetics of the inhibition of neutrophil elastase by α_1PI

Elastase, succinylmethylester-$(Ala)_2$-Pro-Val-p-nitroanilide and α_1PI were rapidly added in sequence to a spectrophotometer cuvette containing 0.2 M Tris-HCl with variable proportions of "smoke solution". The final concentrations of the three reactants were : 0.9×10^{-7}M, 7.6×10^{-4}M and 0.9×10^{-7}M, respectively. The substrate hydrolysis was recorded as described above.

Elastolysis in the presence of α_1PI

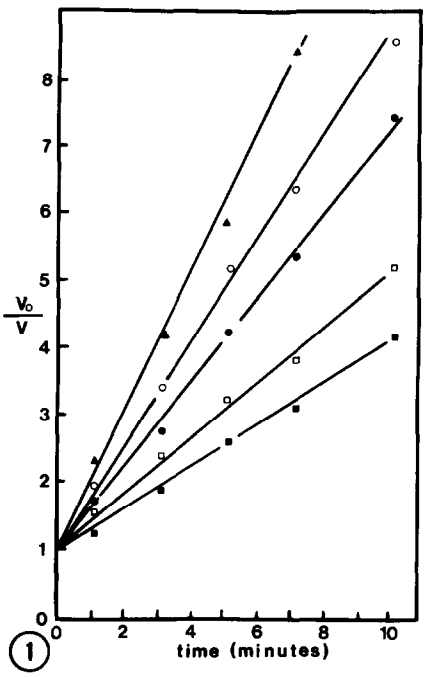
Elastase was added to a mixture of remazol-brilliant-blue elastin and α_1PI and the rate of elastin solubilization was measured. In a typical experiment, 30 mg of labeled elastin were suspended in 3 ml of 0.02 M Tris-HCl pH 8.0 containing a given concentration of "smoke solution". The reaction was started by adding aliquots of α_1PI and elastase in sequence. The suspension was then incubated at 37°C under continuous stirring. Every 30 min, a 300 µl sample was removed from the medium, acidified with 15 µl of 1 M acetate buffer pH 4.5 and centrifuged at 4,000 x g for 10 min. The absorbancy of the supernatant was then read at 595 nm to evaluate the amount of soluble elastin peptides. The absorbancy versus time curves were used to calculate the rate of elastolysis. There curves were linear for at least 4 h. whatever the reaction mixture (data no shown).

RESULTS

Effect of cigarette smoke on the rate of inhibition of pancreatic elastase by α_1PI.

Equimolar concentrations of elastase and α_1PI were reacted for selected time intervals before addition of substrate. If v_0 and v are the rate of substrate hydrolysis at t = 0 and at any time t, respectively, the integrated form of the second-order association rate equation (5) may be written as : $v_0/v = 1 + (E°).k_{ass}.t$, where (E°) is the total elastase concentration. The association rate constant k_{ass} was measured in the absence and in the presence of increasing concentrations of "smoke solution". Satisfactory second-order plots were obtained in all cases (figure 1). It can be seen that cigarette smoke gradually decreases the slope of the curves. The k_{ass} values derived from figure 1 were plotted as a function of the percentage of "smoke solution" present in the reaction mixture (figure 2). k_{ass} decreases linearly from $2.2 + 0.1 \times 10^5$ M^{-1} s^{-1} (absence of "smoke solution") to $0.6 + 0.25 \times 10^5$ M^{-1} s^{-1} (93 % of "smoke solution). Control experiments showed that cigarette smoke had no effect on the elastase-catalyzed hydrolysis of Suc-$(Ala)_3$-NA. The observed decrease in k_{ass} is therefore not an artifact due an effect of cigarette smoke on the enzyme-substrate system.

It has been shown that cigarette smoke is able to oxidize α_1PI, on the one hand (3) and that the oxidized inhibitor is fully inactive on porcine pancreatic elastase, on the other hand (5). It might therefore be argued that our observed decrease in k_{ass} simply results from a partial oxidation of α_1PI, i.e. a decrease in the effective concentration of inhibitor. We have therefore run control experiments in which equimolar quantities (10^{-7}M) of elastase and α_1PI were reacted in the presence of 17 %, 50 % and 97 % of "smoke solution" for a time sufficient to ensure their complete association. The residual enzymatic activities of these mixtures were 0 %, 3 % and 6 %, respectively. This shows that even with the highest concentration of "smoke solution", only



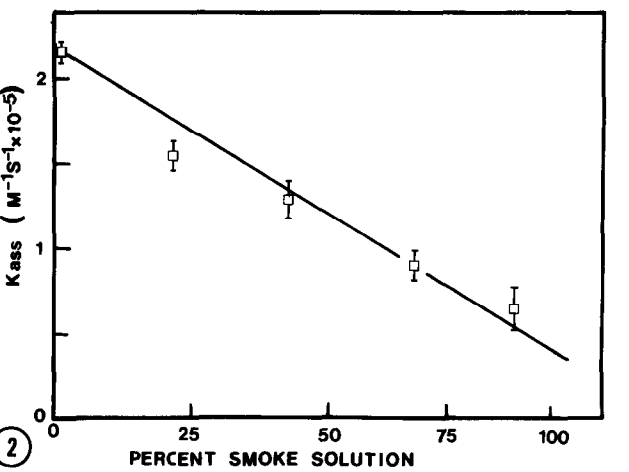


Figure 1 - Time dependency of the inhibition of porcine pancreatic elastase (0.9×10^{-7} M) by an equimolar concentration of α_1-proteinase inhibitor in the absence (▲) and the presence of 20 % (o), 45 % (●), 70 % (□) and 90 % (■) of aqueous "smoke solution". The data are plotted in accordance with the second-order linear equation shown in the text.

Figure 2 - Dose-response curve for the effect of cigarette smoke on the rate constant for the inhibition or porcine pancreatic elastase by α_1-proteinase inhibitor (k_{ass}). Each value of k_{ass} was determined in triplicate. The bars indicate the standard deviations.

about 6 % of α_1PI is oxidized. This is not surprising since our experiments were performed without preincubating α_1PI with "smoke solution" whereas a minimum of 20 min of preincubation was used to observe smoke-induced oxidation of α_1PI (3). Lastly, we wish to add that the second-order plot we use (figure 1) is linear only if the effective concentration of inhibitor is equal to that of the enzyme. As determined from separate experiments, the plot becomes significantly non-linear if the α_1PI concentration is 20 % lower or higher than that of elastase. Since linear plots were repeatedly observed, we may use this observation as an additional proof that the smoke-induced decrease in k_{ass} is not due to the presence of oxidized α_1PI in the reaction mixtures.

Effect of cigarette smoke on the solubilization of elastin by pancreatic elastase in the presence of α_1PI

To study the biological consequence of the association rate-depressing effect of cigarette smoke, we have run experiments in which α_1PI and elastin were allowed to compete for the binding of elastase. The elastin-elastase

interaction was monitored by measuring the rate of elastolysis. The table shows that the reaction of pancreatic elastase with a 1.3-fold molar excess of α_1PI in the presence of elastin does not yield full inhibition of the enzyme, an observation that is similar to that reported for neutrophil elastase (17). The presence of "smoke solution in the test system, renders the inhibitor significantly less potent : in the presence of 50 % "smoke solution", α_1PI is almost unefficient as an elastase inhibitor. This is a consequence of the smoke-induced decrease in the rate of elastase and α_1PI association.

Effect of cigarette smoke on the inhibition of neutrophil elastase by α_1PI

The inhibition of neutrophil elastase by α_1PI is too fast to be monitored by conventional techniques (5). We therefore allowed the inhibitor to react with elastase in the presence of substrate and recorded the time course of product formation. After about 60 s. the latter reached an asymptote corresponding to the complete inhibition of elastase by α_1PI (data not shown). The curves recorded in the absence and the presence of variable volumes of "smoke solution" (up to 20%) were essentially superimposable, indicating that low amounts of cigarette smoke do not significantly decrease k_{ass} (with our substrate-displacement technique, a decrease in k_{ass} would have been easily diagnosed by both the increase in the time required for the substrate product to reach the asymptote and the decrease of the asymptotic product concentration). Negative results were also obtained when α_1PI and elastin were allowed to compete for the binding of elastase (Table 1). We could not use more than 20 % "smoke solution" because neutrophil elastase became insoluble at higher concentrations.

DISCUSSION

Cigarette smoke contains so many components that it may exert non-oxidative effects on biological macromolecules. As a matter of fact, non-oxidative inhibition of the lysyloxidase-catalyzed cross-linking of elastin (18) and of Factor XIII a (19) has recently been reported. The smoke-induced decrease of the rate constant for the inhibition of pancreatic elastase by α_1PI, demonstrated in this paper, is another example of a non-oxidative effect of cigarette smoke on a biological system. The linear dose-effect relationship (fig.2) suggests that this effect is very specific. It is difficult to speculate on the mechanism of action of smoke on the enzyme-inhibitor interaction. Since no change in elastase activity on either synthetic or natural substrate could be detected, it might be inferred that smoke acts on α_1PI itself. Cigarette smoke contains α-dicarbonyl compounds (21) which may react with a limited number of arginine or lysine groups of α_1PI and change the conformation of the protein in a way such that the enzyme-inhibitor interacteration is decreased in rate but not hindered.

Table 1

Effect of cigarette smoke on the elastase-catalysed solubilization of elastin in the presence of α_1-proteinase inhibitor

COMPOSITION OF REACTION MEDIUM[a]			rate of elastolysis ($A_{595} \cdot h^{-1}$)	% inhibition of elastase
elastase	% smoke solution	α_1PI[b]		
PE[c]	0	-	.130	0
PE	0	+	.077	41
PE	50	-	.130	0
PE	17	+	.097	25.5
PE	50	+	.117	10
NE[d]	0	-	.083	0
NE	0	+	.020	76
NE	20	-	.083	0
NE	20	+	.020	76

[a] the reagents were added in the following sequence : remazol-brilliant-blue-elastin + buffer (with or without "smoke solution") + α_1PI (when present) + elastase

[b] molar α_1PI concentration = 1.3 x molar elastase concentration ; - : absent, + : present in reaction medium

[c] porcine pancreatic elastase ; final concentration : .117 µM

[d] human neutrophil elastase ; final concentration : .42 µM

It is worthwhile noticing that a relatively small decrease in k_{ass} yields a rather dramatic increase in the rate of elastolysis when the inhibitor and the substrate are faced with the enzyme as is the case *in vivo*. This finding is of general interest and shows that *in vivo* proteolysis might be controlled by subtle changes in enzyme-inhibitor association or dissociation rate constants. The pathological relevance of our findings is difficult to ascertain as yet because we could not use concentations of "smoke solution" higher than 20 % in the experiments with neutrophil elastase.

The functional activity of biological fluids α_1PI is commonly measured by pancreatic elastase inhibition (9). Before addition of substrate, elastase and the biological fluid must be preincubated long enough to ensure complete association of elastase with α_1PI. If the fluid contains components that decrease k_{ass}, the incubation time determined with pure α_1PI will not be

sufficient. Hence, the concentration of active α_1PI will be underestimated. Bronchoalveolar lavage fluids from smokers are clearly yellow and certainly contain cigarette smoke components. It is not unlikely that these components may be responsible for the reported findings that lung α_1PI from smokers is less active than that from nonsmokers (7,8).

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